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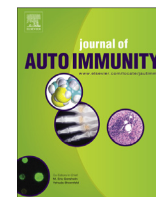

Engrafting human regulatory T cells with a flexible modular chimeric antigen receptor technology

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As regulatory T cells (Tregs) play a fundamental role in immune homeostasis their adoptive transfer emerged as a promising treatment strategy for inflammation-related diseases. Preclinical animal models underline the superiority of antigen-specific Tregs compared to polyclonal cells. Here, we applied a modular chimeric antigen receptor (CAR) technology called UniCAR for generation of antigen-specific human Tregs. In contrast to conventional CARs, UniCAR-endowed Tregs are indirectly linked to their target cells via a separate targeting module (TM). Thus, transduced Tregs can be applied universally as their antigen-specificity is easily adjusted by TM exchange. Activation of UniCAR-engrafted Tregs occurred in strict dependence on the TM, facilitating a precise control over Treg activity. In order to augment efficacy and safety, different intracellular signaling domains were tested. Both 4-1BB (CD137) and CD28 costimulation induced strong suppressive function of genetically modified Tregs. However, in light of safety issues, UniCARs comprising a CD137-CD3ζ signaling domain emerged as constructs of choice for a clinical application of redirected Tregs. In that regard, Tregs isolated from patients suffering from autoimmune or inflammatory diseases were, for the first time, successfully engineered with UniCAR 137/ζ and efficiently suppressed patient-derived effector cells. Overall, the UniCAR platform represents a promising approach to improve Treg-based immunotherapies for tolerance induction.

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1. Introduction

In the past two centuries, regulatory T cells (Tregs) were characterized as an essential component of the human immune system that significantly contributes to tolerance induction and immune homeostasis [1]. Due to their immunosuppressive properties, Tregs possess an enormous potential for treatment of autoimmune or

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chronic-inflammatory diseases, but also for prevention of alloreactive immune responses within the course of hematopoietic stem cell or organ transplantation [2,3]. Accordingly, the adoptive transfer of *ex vivo* expanded, polyclonal Tregs into patients with Graft-versus-Host Disease (GvHD) [4,5] or Type 1 diabetes [6,7] already achieved first promising results. However, several preclinical mouse models clearly demonstrate the superior functionality of antigen-specific Tregs to polyclonal cells [8–12]. On the downside, due to their low frequency in peripheral blood the isolation of human Tregs with a distinct auto- or alloreactivity proves to be technically difficult and time-consuming.

Originally designed for the treatment of malignant diseases, chimeric antigen receptors (CARs) represent a powerful tool to activate T cells antigen-specifically against a target antigen of choice [13,14]. Conventional CAR constructs consist of an extracellular antibody-based binding moiety that is fused to an intracellular T cell activating signaling domain via a transmembrane hinge region. Direct cross-linkage of the genetically modified T cell to the antigen-expressing target cell via the CAR ultimately results in T cell activation and induction of effector functions. However, the *in vivo* activity of CAR-equipped T cells cannot be controlled properly [15,16]. In light of this major drawback of conventionally used CARs, in 2014 we presented a novel modular and flexible CAR system that splits the antigen binding and signaling unit of CARs into two separate components [17]. The so-called UniCAR represents a second generation signaling CAR construct but does not bind directly to target cells. Instead, the interaction is mediated via a targeting module (TM) that contains an antigen-binding moiety and a small peptide epitope which is recognized by the UniCAR. Hence, UniCAR-armed T cells are silent until they encounter the TM that mediates their cross-linkage to target cells. Since 2014, the functionality of this novel switchable UniCAR system has been demonstrated for retargeting of conventional T cells (Tconvs) against various tumor-associated antigens including prostate stem cell antigen (PSCA), prostate-specific membrane antigen, CD33, CD123, EGFR, and GD2 both *in vitro* and *in vivo* [18–21]. Yet, it remains to be shown whether the UniCAR platform technology can also be applied for an antigen-specific redirection of Tregs. Besides, no studies have been conducted so far testing the influence of CARs with different costimulatory signaling domains on Treg functionality. To shed light on this issue, we designed UniCARs either lacking a costimulatory signal (UniCAR ζ) or comprising the signaling domain of human CD28 (UniCAR 28/ζ) or CD137 (UniCAR 137/ζ), respectively.

In this study, we demonstrate that not only Tconvs but also Treg cells isolated from either healthy donors or patients with an active inflammatory disease can be successfully modified and antigen-specifically activated using the UniCAR technology both *in vitro* and/or *in vivo*. Furthermore, differences in Treg properties were observed in dependence on the intracellular signaling domain engrafted into the cells.

2. Materials and methods

2.1. Cell lines

All cell lines were purchased from the American Type Culture Collection (ATCC), cultured at 37 °C in a humidified atmosphere (5% CO_2). The prostate cancer cell line PC3 (ATCC CRL-1435) was transduced with PSCA as previously described [22] and kept in RPMI 1640 completed with 10% FCS, 100 μg/ml penicillin/streptomycin, 1% non-essential amino acids, 1 mM sodium pyruvate and 2 mM N-acetyl-L-alanyl-L-glutamine (all purchased from Biochrom, Berlin, Germany). The human embryonic kidney cell line HEK 293T (ATCC CRL-11268) used for production of virus particles and the TM-secreting murine fibroblast cell line 3T3 (ATCC CRL-1658) were grown in DMEM (ThermoFisher Scientific, Schwerte, Germany) supplemented with 10% FCS, 100 μg/ml penicillin/streptomycin and 1% non-essential amino acids.

2.2. Design of UniCAR constructs

Generation and structural design of the UniCAR 28/ζ construct was already described elsewhere [18,19]. In brief, it contains an extracellular scFv-derived from an αLa 5B9 mAb [23]. Hinge, transmembrane and signaling domain originate from human CD28 and are followed by a human CD3ζ signaling moiety [24]. For extracellular detection of the UniCAR, a peptide epitope tag (E7B6, aa sequence EKEALKKIIEDQQESLNKW) derived from the human La/SS-B protein is introduced between scFv and CD28 hinge region [19,24].

The novel constructs UniCAR stop, UniCAR ζ and UniCAR 137/ζ were obtained by ligation of three individual fragments. Fragments 1 and 2 were gained by digesting UniCAR 28/ζ with *Nhe*I/*Ksp*AI and *Bam*HI/*Nhe*I, respectively. For construction of UniCAR stop, the required gene proportion was amplified from UniCAR 28/ζ by PCR using an Advantage® HF2 PCR Kit (Clontech Laboratories, Inc., Mountain View, CA, USA) as well as the primers #1 (5′-CGTTAACCAAACAGATATTTAC) and #2 (5′-CGGATCCCCTAGACCTCTTAGATCTG). The resulting PCR product was subcloned into pGEM®-T Easy vector (Promega GmbH, Mannheim, Germany) which was subsequently digested with *Ksp*AI and *Bam*HI leading to fragment 3A. In case of UniCAR ζ, the open reading frame of fragment 3B was cut out of a cloning vector available in the lab (unpublished sequence) by using the restriction enzymes *Ksp*AI and *Bam*HI. For generation of UniCAR 137/ζ the gene sequence encoding fragment 3C including the signaling domain of human CD137 (amino acid sequence taken from UniProt) was synthesized by Eurofins Genomics (Ebersberg, Germany). The delivered vector was also digested with *Ksp*AI and *Bam*HI resulting in the required gene proportion. By ligation of the aforementioned fragments 1, 2 and 3A, 3B or 3C, respectively, the expression vectors p6NST60-UniCAR stop, p6NST60-UniCAR ζ and p6NST60-UniCAR 137/ζ were generated. Structural layout of newly generated UniCAR constructs is accurately described in the results part.

2.3. Design, expression and purification of the recombinant αPSCA TM

Design, cloning and purification of αPSCA TM with a fused E5B9 tag (aa sequence SKPLPEVTDEY) was already described elsewhere [25,26]. Briefly, culture supernatant of the production cell line 3T3 was harvested and TM was purified by Ni-NTA affinity chromatography (Qiagen, Hilden, Germany). The bound αPSCA TM was eluted with ice-cold PBS containing 350 mM imidazole and 150 mM NaCl followed by dialysis overnight against PBS. To assess purity and concentration, the eluted αPSCA TM was analyzed by SDS-PAGE and Coomassie brilliant blue G250 staining in comparison to a BSA standard using Image Lab™ software (Bio-Rad Laboratories GmbH, Munich, Germany).

2.4. Flow cytometry

Flow cytometric analysis was performed using a MACSQuant® Analyzer and MACSQuantify® software (Miltenyi Biotec, Bergisch Gladbach, Germany). mAbs against human CD3 (BW264/56), CD4 (VIT4), CD8 (BW135/80), CD25 (4E3), CD45RA (T6D11), CD69 (FN50), CD127 (MB15-18C9), granzyme B (REA226) and IgG1 (isotype control, IS5-21F5) were purchased from Miltenyi Biotec. A goat anti-mouse IgG F(ab')2 antibody was obtained from Immunotech

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